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Research Article

Genetic structure and microsatellite-based genetic variation influenced by habitat fragmentation in pudu deer (*Pudu puda*) from southern Chile

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Abstract

Landscape alterations have strong impacts on the distribution of genetic variation within and between populations, and understanding these effects can provide insights for design conservation strategies. We analyzed 20 georeferenced individuals of pudu deer (*Pudu puda*) of three populations from southern Chile to obtain genetic diversity and distance parameters based on five polymorphic microsatellite loci. The genetic population structure was assessed by using STRUCTURE 2.3.4 and the effective population sizes (Ne) with NeEstimator 2.0 software. Habitat fragmentation metrics were also analyzed to correlate these data with the genetic variation focused to assess the effect of native forest fragmentation on the genetic structure of pudu deer. The mean number of alleles ranges from 2.8 to 4.2, while the mean number of private alleles ranged from 0.4 to 1.2. The mean observed heterozygosity ranged from 0.423 to 0.547. All populations displayed significant deficits of heterozygotes (F_{IS} = 0.091-0.189, P < 0.0001). The F_{ST} on the whole populations and loci was low (mean F_{ST} = 0.0942) and without statistical significance (P > 0.05), indicating no genetic differentiation among populations. The STRUCTURE analysis did not reveal a population structure, and the NeEstimator found a markedly low Ne in a population compared to other analyzed populations. Multiple regression analysis indicates that two fragmentation metrics, namely, the proportion of the landscape covered by forest (PLAND) and the patch density (PD), negatively and significantly affected the number of alleles per locus as well as the observed heterozygosity (PLAND, β = -0.8740, P < 0.05; PD, β = -0.6594, P < 0.05). This result suggests that a greater degree of habitat fragmentation contributes to a decrease in genetic variation of pudu deer. Therefore, maintaining the native forest continuity across the distribution range of pudu deer could contribute to preserving the genetic diversity, enhancing the long-term survival of this mammal.

Key words: Conservation genetics, genetic variation, habitat fragmentation, land cover, landscape genetics, microsatellite loci, population structure, pudu deer

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Introduction

Pudu deer, *Pudu puda*, is endemic to southern South America and is characterized as being one of the smallest deer in the world due to its short shoulder height (30–40 cm) and small body weight (< 15 kg) (Jiménez 2010). This species is distributed throughout Chile from 35°10'S to 46°45'S, i.e., from the Maule to Aysen regions (Jiménez 2010), occupying an area of 128,278 km^2 according to the International Union for Conservation of Nature (IUCN). Pudu deer characteristically inhabit pristine temperate rainforests, particularly in areas of dense understorey growth and native bamboo thickets (Eldridge et al. 1987; Meier and Merino 2007), although some reports also indicate that it can be found in secondary and disturbed forest habitats (Jiménez 2010).

The current conservation status of pudu deer is near threatened according to the IUCN. This conservation status is related to different threats that have affected the viability of the species, mostly linked to the expansion of human activities during recent decades, such as native forest loss and fragmentation, predation by domestic dogs, competition with exotic species, and poaching activities (Miller et al. 1973; Wemmer et al. 1998; Silva-Rodríguez et al. 2010; Silva-Rodríguez and Sieving 2012; Jiménez and Ramilo 2013). In the case of native forest loss and fragmentation, it has been intensive in southern Chile and, therefore, is a matter of particular concern for pudu deer conservation. For example, in Osorno and Llanquihue Provinces the area planted with exotic tree species between 2006 and 2013 increased significantly (+20.6% and +61.3%, respectively), and a large part of this growth occurred at the expense of native forest (CONAF-UACh 2014). Another detrimental effect on native forest integrity has been its gradual replacement by grasslands and bushes, as occurred in areas of Los Ríos and Los Lagos regions (25% and 27%, respectively), according to a study of historical reconstruction of vegetational cover (Lara et al. 2012). This phenomenon is more accentuated in the central valleys, located between the Andean and Coastal mountain ranges (Miranda et al. 2017), where there is greater agricultural and forestry activity. Thus, the conservation of pudu deer in southern Chile is a matter of public interest that requires combined efforts in several research areas to assess the effect of different threats affecting their survival in the natural environment (Silva-Rodríguez et al. 2011).

Landscape genetic studies integrate population genetics, landscape ecology, and spatial statistics to develop and test predictions regarding the influence of landscape features on spatial population genetic structure and gene flow (Manel et al. 2003; Storfer et al. 2007). These analyses may identify certain landscape-scale variables as suitable macrohabitat conditions and indicate features that may act as barriers to or facilitators of gene flow. Moreover, the incorporation of landscape genetics into conservation planning may inform more targeted management approaches to promote species conservation than those that simply use existing information about habitat preferences (Segelbacher et al. 2008). There are several examples in different mammal species that support its aforementioned utility (Bani et al. 2017; Thatte et al. 2018), including some deer species (Coulon et al. 2006; Mucci et al. 2012; Burkart et al. 2016). In this study, we used an individual-based approach because it has the advantage of covering a wide geographic area using a small number of individuals (Rousset 2000; Richardson et al. 2016). Thus, this strategy permits high-resolution sampling

within an area or the inclusion of a wider sampling area of the landscape. Simulations suggest that the ability to detect landscape effects is similar or better using individual-based sampling as group sampling (Luximon et al. 2014). Individual-based approach has been applied to some deer species, such as the roe deer (Coulon et al. 2004, 2006) and white tailed deer (Scribner et al. 2005), to test its ability to detect weak population genetic structure for a non-clustered distribution of individuals across a fragmented landscape (Coulon et al. 2006).

Available genetic studies performed in pudu deer from Chile have addressed the description of genetic markers (Marín et al. 2014), the phylogenetic relationship among populations across the distribution range (Fuentes-Hurtado et al. 2011; Cabello 2019) and the genetic divergence and demography between provinces (Colihueque et al. 2022). Despite this progress, until now, no genetic studies have focused on analyzing the influence of landscape features on the population genetic structure of the species. This issue is interesting to address given that the genetic structure of pudu deer populations in southern Chile is likely to be affected by native forest loss and fragmentation as a consequence of the expansion of human activities in this area (Echeverría et al. 2006, 2007; Miranda et al. 2017). Presumably, these factors may contribute to genetic differentiation between populations, since fragmented populations are expected to differentiate more because migration between them is impaired. In addition, genetic variation loss may also occur in fragmented populations because genetic variation is reduced as a consequence of a decrease in population effective size and genetic drift or by the impairment of gene flow with other populations (Höglung 2009). A reduction in genetic variation, estimated as an overall decrease in allelic diversity, allelic richness, observed heterozygosity, and expected heterozygosity, has been well supported in many mammalian species living under conditions of high habitat fragmentation (Lino et al. 2019). Likewise, in deer species, landscape characteristics have also been identified as important factors that can affect their genetic structure. In fact, in roe deer (*Capreolus capreolus*), a combination of landscape features, such as rivers and canals, can lead to population differentiation (Coulon et al. 2006), and in fragmented woodland areas, gene flow within the roe deer population is influenced by the connectivity of the landscape (Coulon et al. 2004). In the same species, Breyne et al. (2014) provided evidence that landscape features related to transportation infrastructure may affect its genetic variation by hindering gene flow.

In this study, we obtained a number of genetic diversity and distance parameters of pudu deer that inhabit three provinces in southern Chile using microsatellite loci to examine their genetic variation and the population genetic structure. We use genotypes from georeferenced individuals to evaluate the impact of native forest fragmentation on the genetic variation using a landscape genetics approach. The latter objective was performed by applying a stepwise multiple regression analysis to assess whether the combinations of habitat fragmentation metrics affected the variation in another genetic diversity parameter. We expected that native forest fragmentation affects the population genetic structure of pudu deer, which can be reflected in a close relationship between reduced genetic variation levels as a function of augmented habitat fragmentation. Such association is expected because the fragmented habitat imposes constraints on dispersal patterns of species that may reduce or interrupt gene flow, and as a result, populations become genetically more structured and less diverse (Lino et al. 2019; Brunke et al. 2020).

Methods

Study area

The area used to conduct the landscape genetic analysis of pudu deer corresponds to the region located between 40° and 43° south in Chile. This geographic area covers 32,936.2 km^2 and includes the provinces of Osorno (40°35'00"S, 73°10'00"W; 9,246.6 km^2), Llanquihue (41°20'00"S, 72°50'00"W; 14,706.8 km^2) and Chiloé (42°30'0"S, 74°0'0"W; 8,982.8 km^2), the latter composed by the archipelago of Chiloé (Fig. 1). This region contains a substantial remnant of native temperate rainforest that is mostly distributed across the coastal and Andes mountain ranges (Miranda et al. 2017) and covers large portion of each Province: 42.9% in Osorno, 54.5% in Llanquihue and 68.3% in Chiloé (CONAF-UACh 2014). The climate in this region is warm temperate and rainy with a Mediterranean influence and mean annual precipitation of 2,490 mm and temperature of 12.0 °C (Errazuriz et al. 2000). The Coastal Mountain range located in this area is characterized by an average height of 500 masl, which tends to gradually decrease in the southern part of the range (Ramírez and San Martín 2005; Villagrán and Armesto 2005). Meanwhile, the Andes Mountains have higher elevations, averaging 1,500 masl, with some elevations above 3,000 masl (Garreaud 2009). Both mountain ranges exhibit predominately temperate laurifoliar rainforest vegetation formations, including the Valdivian, North Patagonian and Subantarctic types (Villagrán and Hinojosa 2005). The temperate rainforest of Chile encompasses the Valdivian rainforest ecoregion, which has been listed among the most endangered ecoregions of the world and has a critical conservation status (Dinerstein et al. 1995; Olson and Dinerstein 2008; Miranda et al. 2017). In addition, the Valdivian rainforest ecosystem is considered a biodiversity hotspot and is, therefore, a region of high conservation priority (Ormazabal 1993; Myers et al. 2000; Smith-Ramírez 2004). However, despite the importance of these forest formations for the conservation of biodiversity in southern Chile, they have undergone a rapid rate of anthropogenic degradation (Myers et al. 2000). This change had impacted the level of forest loss and fragmentation in this geographical area over time since, for example, in representative landscapes of Llanquihue and Chiloé Provinces, the number of patches of native forest per 100 hectares (i.e., patch density) increased significantly between 1976 and 1999 (Echeverría et al. 2008), which reflects an increasing fragmentation level. This phenomenon is more marked in the central valleys located between the Andean and Coastal mountain ranges (Miranda et al. 2017), where there are greater levels of agricultural and forestry activity.

Sampling

Pudu deer individuals were collected between 2014 and 2019 in the Osorno (n = 5), Llanquihue (n = 9) and Chiloé (n = 6) Provinces (Table 1, Fig. 1). The majority of the samples consisted of skin and muscle collected from dead animals, whose carcasses were donated by different institutions to our laboratory (Museo de Historia Natural, Purranque; Parque Nacional Puyehue, Osorno; Sitio Paleontológico de Pilauco, Osorno; Centro de Reproducción del pudu, Osorno). No further samples could be included in this study as pudu deer carcasses are often scarce and, when they were available, most of them lacked precise

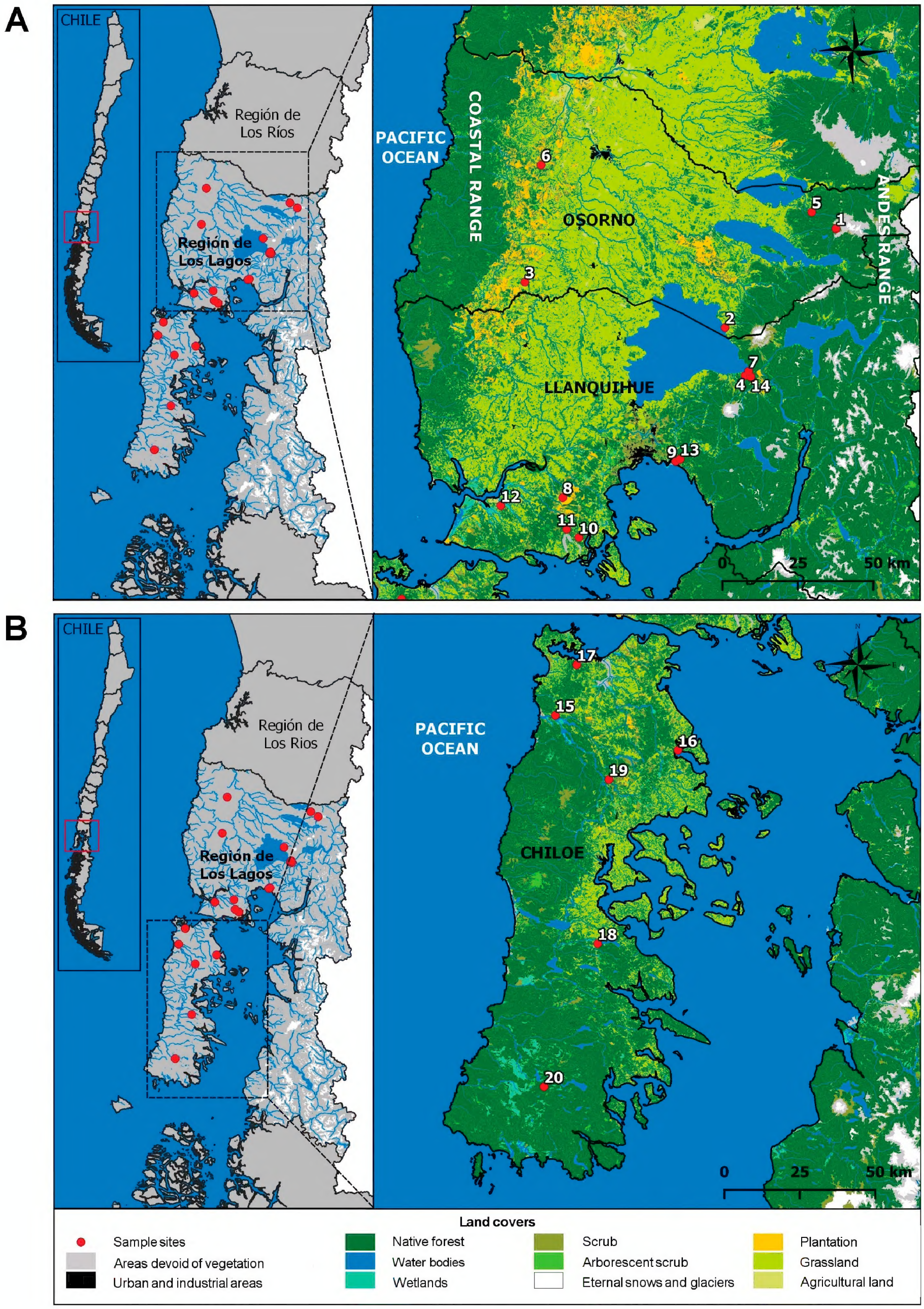

Figure 1. Sampling sites of pudu deer in southern Chile. Sampling sites in Osorno and Llanquihue Provinces (**A**); sampling sites in Chiloé Province (**B**). The red dots indicate sampling sites within each province. The land cover categories in different colored rectangles are identified below map. Sampling site numbers are detailed in Table 1.

Table 1. Samples of pudu deer from southern Chile used in this study.

Site number	Specimen ID	Latitude, Longitude	Date	Deposition	Province	Location
1	1279ULA	40.7820°S, 72.2114°W	2014	LBMULA	Osorno	Parque Nacional Puyehue, Puyehue
2	1330ULA	41.0869°S, 72.6360°W	2016	LBMULA	Osorno	Las Cascadas, Puerto Octay
3	1331ULA	40.9644°S, 73.4288°W	2016	LBMULA	Osorno	Colonia Zagal, Purranque
4	1528ULA	41.2082°S, 72.5383°W	2018	LBMULA	Llanquihue	Ensenada, Puerto Varas
5	1505ULA	40.7353°S, 72.3081°W	2016	LBMULA	Osorno	Parque Nacional Puyehue, Puyehue
6	1515ULA	40.6142°S, 73.3732°W	2017	LBMULA	Osorno	Puaucho, San Juan de la Costa
7	1522ULA	41.6050°S, 73.2623°W	2019	LBMULA	Llanquihue	Chinquihue, Puerto Montt
8	1520ULA	41.2082°S, 72.5383°W	2018	LBMULA	Llanquihue	Ensenada, Puerto Varas
9	1524ULA	41.4891°S, 72.7953°W	2018	LBMULA	Llanquihue	Puerto Montt
10	1525ULA	41.7231°S, 73.1951°W	2018	LBMULA	Llanquihue	Calbuco
11	1526ULA	41.6992°S, 73.2445°W	2018	LBMULA	Llanquihue	Guayún, Calbuco
12	1527ULA	41.6324°S, 73.5083 °W	2018	LBMULA	Llanquihue	Maullín
13	1529ULA	41.4891°S, 72.7953°W	2019	LBMULA	Llanquihue	Puerto Montt
14	1532ULA	41.2082°S, 72.5383°W	2017	LBMULA	Llanquihue	Ensenada, Puerto Varas
15	1516ULA	42.0439°S, 73.9679°W	2019	LBMULA	Chiloé	Chepu, Ancud
16	1517ULA	42.1426°S, 73.4743°W	2019	LBMULA	Chiloé	Degañ, Quemchi
17	1518ULA	41.8991°S, 73.8893°W	2019	LBMULA	Chiloé	Pauldeo, Ancud
18	1519ULA	42.7231°S, 73.7855°W	2019	LBMULA	Chiloé	Chonchi, Chonchi
19	1523ULA	42.2332°S, 73.7499°W	2019	LBMULA	Chiloé	Butalcura, Ancud
20	1531ULA	43.1517°S, 73.9954°W	2019	LBMULA	Chiloé	Cotao, Quellón

Coordinates data were recovery from GeoNames (http://www.geonames.org/) according to locality location;
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collection sites, a piece of information that is crucial for landscape genetic analyses. In addition, due to legal restrictions it was not possible to include more samples in the analysis, since pudu deer is currently classified as vulnerable by the Chilean Ministry of Environment. Tissue samples of each individual were fixed in 80% ethanol. In addition, blood samples were also collected from captive individuals at the Centro de Rehabilitación de Fauna Silvestre (CEREFAS) of the Universidad San Sebastián, Puerto Montt, Chile. The sampling sites of each individual were georeferenced using a portable GPS device; otherwise, these data were obtained based on locality names using the GeoNames platform (http://www.geonames.org/). Moran's I index was also calculated to measure the overall spatial autocorrelation of the dataset, based on the estimation of observation independence within a dataset (Moran 1950).

DNA extraction and genotyping

DNA was extracted from the fixed tissue using the phenol–chloroform method, as described in Taggart et al. (1992). Extracts were standardized at 100 ng/µL using Tris–EDTA buffer at pH 8.0. We used six polymorphic microsatellite loci, named RT27, N, BM6506, BM203, BBJ2 and CERVID14, a set of markers that have been recommended for pudu deer genotyping due to its high levels of allelic richness (5 to 11 alleles) and observed heterozygosity (0.400 to 0.785) compared with other less polymorphic microsatellite loci available for species (Marín et al. 2014) (Suppl. material 1). We used the most polymorphic microsatellite loci because this strategy is recommended for improving the detection of genetic diversity in populations when a small number of loci are analysed (Arthofer et al. 2018). In fact, simulations support that when microsatellite loci

with higher allelic richness are used, they perform better at monitoring the genetic diversity of populations than do less polymorphic loci (Arthofer et al. 2018). Microsatellite loci were initially isolated from other deer and artiodactyl species and successfully cross-amplified in pudu deer. In the pudu deer genome, it is unknown whether these loci belong to different linkage groups, although genome assembly data at the chromosome level from the original species where these markers were isolated suggest that most of them are probably unlinked (Suppl. material 1). The microsatellite locus Cervid14 was excluded from the analyses because it was not possible to obtain clearly interpretable PCR products. Amplification of the microsatellite loci was carried out by polymerase chain reaction (PCR) using fluorescent primers marked at their 5' forward end with a fluorochrome that gives them specific staining (HEX: green, TAMRA: yellow or FAM: blue). Primers used in this study are provided in Suppl. material 2. The genotype of each individual was determined by reading fluorochrome-labelled PCR fragments through the automatic sequencer ABI 3730XL (Macrogen Inc., South Korea) and using 350ROX fluorochrome (Applied Biosystems) as internal size standards. GeneMapper Software version 5 (Applied Biosystems, Warrington, UK) was used to determine the allele sizes, performing manual checks for scoring errors. The microsatellite amplification conditions were as follows: 0.9 µl of $MgCl_2$ at 50 mM, 0.3 µl of each dNTP at 10 mM, 0.1 µl of Taq polymerase at 5 U/µl, 0.3 µl of primers at 10 µm and 3.0 µl of genomic DNA at 100 ng/µl, in a total volume of 15 µL. For PCR reactions, we used KAPA Taq DNA polymerase, following the manufacturer's recommendations (Kapa Biosystems Inc., USA). The thermal amplification profile was as follows: initial denaturation at 94 °C for 3 min, 10 amplification cycles of 94 °C for 45 s, hybridization at 50 °C–65 °C (-1 °C per cycle) for 45 s; 72 °C for 1 min 30 s; additional 30 cycles with a constant annealing temperature at 50 °C and a final extension at 72 °C for 10 min. Prior to fragment analysis, the microsatellite amplifications were checked in agarose gels at 2% w/v, stained with the SYBR Green I for nucleic acids (Invitrogen, USA) and visualized under UV light in the transilluminator.

Population genetic analysis

Genetic variation of the sampling populations by measuring the number of alleles (Na), allelic richness (Ar), observed heterozygosity (Ho), expected heterozygosity (He), and the inbreeding coefficient (F_{IS}) were investigated using GDA_NT version 1b (Degen 2022). The significance of the F_{IS} was calculated using 10,000 permutations. Single locus Hardy–Weinberg Equilibrium (HWE) with Holm-Bonferroni correction (Holm 1979) was computed using GenAlEx version 6.501 (Peakall and Smouse 2006, 2012). The number of private alleles (Pa), the effective number of alleles (Ne), and the number of locally common alleles (frequency > 5%) occurring in 50% or less of the populations (LCA50) were also calculated. Population genetic differentiation by measuring the F_{ST} (Wright 1965), the standardized F_{ST} (Hedrick 2005), and the population differentiation Dj (Gregorius and Roberds 1986) was calculated using GDA_NT version 1b (Degen 2022). The significance of genetic distances was calculated using 10,000 permutations. The number of alleles and Ho for individuals at each locus were calculated using GDA version 1.1 (Lewis and Zaykin 2001). This data set was used for the landscape genetics examination by using an individual-based

approach. Prior to performing the analyses, each locus was screened for null alleles using MICROCHECKER (van Oosterhout et al. 2004); this examination showed no evidence for null alleles among all loci. Using STRUCTURE software version 2.3.4 (Pritchard et al. 2000, Falush et al. 2003), we inferred population structure by testing K values (number of populations) ranging from one to six, with 50,000 iterations of burn-in and a run length Markov-chain Monte Carlo (MCMC) of 200,000 iterations, and considering ten independent runs of K. These runs were conducted using an ancestry mixture model and locality information priors to improve the detection of structure when genetic structure is weak (Hubisz et al. 2009). We conducted this analysis for the entire dataset. To choose the most likely K value from these analyses, we conducted the ΔK Evanno's index (Evanno et al. 2005) implemented in StructureSelector (Li and Liu 2018). In this analysis, the degree of admixture of populations was evaluated by Dirichlet parameter α, considering that a value of $\alpha > 1$ implies that most individuals are admixed. The estimation of membership coefficients for each individual in each cluster was done by the Q-value parameter. We plotted coefficients of individual membership using the CLUMPAK tool of the StructureSelector (Li and Liu 2018) with 2,000 repeats to account for label switching artefacts and multimodality in each K tested. We also estimated effective population sizes (Ne) using a bias-corrected version of the linkage disequilibrium (LD) method by Waples and Do (2008) as implemented in the NeEstimator v2 software (Do et al. 2014). This approach is based on the rationale that in small populations with few parent individuals, genetic drift will create non-random combinations of alleles of different loci, i.e., LD. To facilitate evaluation of the effects of low frequency alleles on estimates of effective size, because rare alleles may have a high impact on the linkage values, which occur frequently in highly polymorphic markers such as microsatellite loci, the Ne value was calculated under different critical threshold or Pcrit values of 0.05, 0.02 and 0.01. Alleles were excluded only if they occur at a frequency less than Pcrit. Confidence intervals (CIs) based on the jack-knife approach were used to estimate the actual variance around Ne estimates. In general, the LD approach is reliable if effective population sizes are not much larger than 200, the data set is based on 10 or more loci and population sample sizes are 25 or more.

Native forest fragmentation analysis

To associate native forest fragmentation with the genetic variation, we used land use data reported for the Región de Los Lagos that were obtained from the Sistema Integrado de Monitoreo de Ecosistemas Forestales Nativos de Chile (SIMEF) of the Ministerio de Agricultura of the Gobierno de Chile (https://simef.minagri.gob.cl), published in 2013. This shape was obtained from Landsat 8 satellite images and has a minimum mappable area of 0.24 ha for the representation of land use. This shape comprises an 11-category scheme and includes urban and industrial areas, agricultural land, grassland, scrub, arborescent scrub, plantation, native forest, wetlands, areas devoid of vegetation, eternal snows and glaciers, and water bodies. This information was intercepted by each home range of pudu deer defined at each sampling site, which corresponded to a radius of 6.1 km. The home range was obtained by averaging different documented values of the maximum dispersal ability of the species

(Eldridge et al. 1987; Ramilo 1992). This layer was subsequently transformed into a bounding rectangle of 12.2 × 12.2 km, which included a mixture of different land use categories, to perform fragmentation analysis (Fig. 2). Native forest areas across sampling sites varied widely in number (2-202) and size (0.24–11,854.2 ha), which reflects a variable level of fragmentation. In addition, these native forest areas were surrounded by plantations or grasslands and, to a lesser extent, by scrubs' areas. Processing of the layers was performed in QGIS version 3.10.12 (QGIS Development Team 2018) and GRASS version 7 (GRASS Development Team 2016). Vegetation type and fragmentation were determined with the FRAGSTATS version 4.2 program (McGarigal et al. 2002). We calculated five fragmentation class metrics for native forest as follows: 1) the proportion of the landscape covered by forest (PLAND), ranging from no forest (0) to entirely forested (100); 2) patch density (PD), which equals the number of forest patches per 100 hectares, a measure of subdivision that reflects the degree to which the landscape is broken up into disjunct patches; 3) the landscape division index (DIV), ranging from entirely forested (0) to almost entirely non-forested with only a single small forest patch in area as it approaches 1; 4) the splitting index (SPLIT), is equal to 1 when the landscape consists of a single forest patch, considering that this metric increases as the forest is increasingly reduced in area

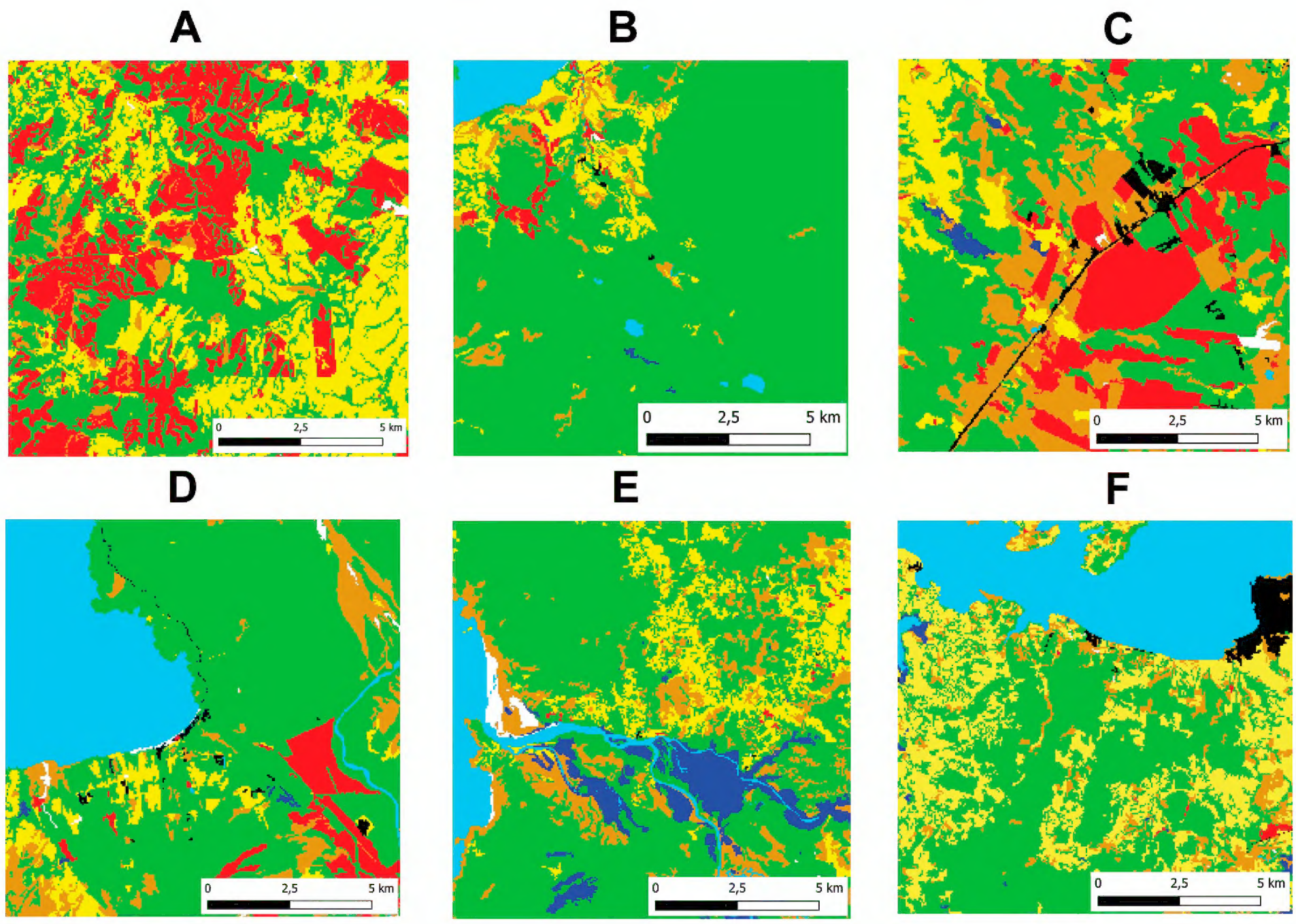

Figure 2. Bounding rectangles of 12.2 × 12.2 km representative of the pudu deer home range, showing the layers of the land use categories used in the fragmentation analysis. Osorno Province: sampling site 1 (**A**), sampling site 5 (**B**); Llanquihue Province: sampling site 7 (**C**), sampling site 8 (**D**); Chiloé Province: sampling site 15 (**E**), sampling site 17 (**F**). Native forest are in green, grasslands in yellow, scrub in brown, plantations in red, water bodies in light blue, wetlands in blue and urban and industrial areas in black. Other land use categories are not shown. Sampling site numbers are detailed in Table 1.

and subdivided into smaller patches; and 5) the number of patches (NP). In addition, four forest cover patch metrics were analyzed: 1) the patch size (SIZE), 2) coefficient of variation of patch size (SIZE_CV), 3) patch proximity index (PROX) and 4) coefficient of variation of patch proximity index (PROX_CV). For the patch proximity index increasing values indicate that a patch of the same type become closer and more contiguous in distribution, i.e., the forest is less fragmented.

Landscape genetic analysis

To assess the relationship between the landscape variables and the genetic parameters, represented by *A* and H_o, we applied a forward stepwise multiple regression analysis to identify the combinations of habitat fragmentation metrics that explained the most variation in the genetic variation of sampling sites. For this analysis we considered all the sampled pudu deer individuals as statistical units. In addition, the SPLIT, NP, SIZE and PROX variables were scaled using a $\log_{10}$ function. Prior to the analysis the DIV variable was arcsine-transformed and the SIZE_CV variable was square root transformed to ensure better conformity to the assumptions of normality and homogeneity of variance. To assess the aforementioned assumptions for these variables we used the Kolmogorov–Smirnov and Levene's tests, respectively. Stepwise multiple regressions are a useful statistical tool for identifying combinations of independent variables that explain the most variation in another dependent variable (Sokal and Rohlf 2009). The criteria used for variable selection during forward stepwise regression were an F-statistic above 3.84 as the entrance condition and an F-statistic value of 2.71 as an exit criterion. The residuals of the final models were explored to verify the assumptions of normality, homogeneity and linearity. The absence of multicollinearity between independent variables was also verified through the variation inflation factor (VIF), based on the tolerance value of each variable, according to the formula VIF = 1/tolerance. When the value of the VIF was more than 5, the multicollinearity was considered serious, and the variable was eliminated from the analysis (Ghani and Ahmad 2010). These statistical analyses were performed using the STATISTICA version 5.1 program. In addition, to control the probability that a true null hypothesis had been incorrectly rejected as a consequence of multiple hypotheses testing, a bootstrap procedure using 2000 bootstrap samples for correction of P-values was applied, a re-sampling method that is recommended for correlated outcomes (Westfall and Young 1993). For this analysis, an $\alpha = 0.05$ was used as the significant threshold. This bootstrap analysis was done using the SPSS version 22 program.

Results

Population genetic analysis

All microsatellite loci were polymorphic (Suppl. material 3). The number of alleles ranged from 2 to 4, from 2 to 4, and from 2 to 7, while the allelic richness varied from 1.8 to 3.3, from 1.6 to 2.9, and from 1.8 to 4.1 in Osorno, Chiloé, and Llanquihue populations, respectively (Table 2). The overall mean number of alleles and allelic richness was 3.4 and 2.7, respectively. The level of genetic diversity, measured as a mean number of alleles and allelic richness, was higher in the population of

Table 2. Genetic population parameters of pudu deer populations from southern Chile.

		Population parameters							HWE test		
Populations	**Locus**	**N**	**Na**	**Ar**	**Ho**	**He**	**F_{IS}**	**P**	**Df**	**Chi-sq**	**P**
Osorno	RT27	5	3	2.6	0.600	0.580	-0.034	0.0001	3	1.050	0.789
	N	5	3	2.8	0.250	0.656	0.619	0.0001	3	4.444	0.217
	BM6506	5	2	2.0	0.000	0.500	1.000	0.0001	1	4.000	**0.046**
	BM203	5	4	3.3	1.000	0.719	-0.391	0.0001	6	6.667	0.353
	BBJ2	5	2	1.8	0.400	0.320	-0.250	0.0001	1	0.313	0.576
	Mean		2.8	2.5	0.450	0.555	0.189	0.0001			
Chiloé	RT27	6	2	1.6	0.200	0.180	-0.111	0.0001	1	0.062	0.804
	N	6	4	3.2	1.000	0.700	-0.429	0.0001	6	3.750	0.710
	BM6506	6	3	2.5	0.333	0.500	0.333	0.0001	3	6.375	0.095
	BM203	6	4	2.9	0.333	0.639	0.478	0.0001	6	6.480	0.372
	BBJ2	6	3	2.3	0.250	0.406	0.385	0.0001	3	8.000	**0.046**
	Mean		3.2	2.5	0.423	0.485	0.131	0.0001			
Llanquihue	RT27	9	2	1.8	0.333	0.278	-0.200	0.0001	1	0.240	0.624
	N	9	5	3.8	0.667	0.778	0.143	0.0001	10	12.667	0.243
	BM6506	9	3	2.5	0.400	0.580	0.310	0.0001	3	2.450	0.484
	BM203	9	7	4.1	0.833	0.764	-0.091	0.0001	21	26.160	0.200
	BBJ2	9	4	3.3	0.500	0.708	0.294	0.0001	6	9.227	0.161
	Mean		4.2	3.1	0.547	0.622	0.091	0.0001			
	Overall mean		3.4	2.7	0.473	0.554	0.137				

N = number of genotyped individuals, Na = number of alleles, Ar = allelic richness, Ho = observed heterozygosity, He = expected heterozygosity, F_{IS} = inbreeding coefficient, HWE = Hardy–Weinberg Equilibrium. Bold indicate the significant values ($P < 0.05$).

Llanquihue (Na = 4.2 and Ar = 3.1) than in the Osorno (Na = 2.8 and Ar = 2.5) and Chiloé (Na = 3.2 and Ar = 2.5) populations (Table 2). Mean values of the effective number of alleles were 2.463, 2.201 and 3.186 in Osorno, Chiloé, and Llanquihue populations, respectively, while the mean number of private alleles were 0.400, 0.800, and 1.200. The mean number of locally common alleles occurring in 50% or less of the populations was zero in all populations, indicating the occurrence of a high frequency of unique alleles within populations (Suppl. material 3).

The mean observed heterozygosity of populations ranged from 0.423 to 0.547, with an overall mean value of 0.473 (Table 2). The observed heterozygosity was generally lower than expected for all loci at all populations. All populations displayed significant deficits of heterozygotes (mean $F_{IS} > 0$), and the heterozygote deficiency was significant ($P < 0.0001$). The mean F_{IS} values were 0.091 in Osorno, 0.131 in Chiloé and 0.189 in Llanquihe (Table 2). This deficit was due to two to three loci. The mean F_{IS} value over all loci was 0.137. Among the analyzed loci, BM6506 locus in Osorno and BBJ2 locus in Chiloé populations showed significant deviation from HWE ($P < 0.05$). The mean F_{ST} and the standardized F_{ST} on the whole populations and loci were 0.0942 and 0.2677 (Table 3). Both F_{ST} values were not significantly different between populations ($P > 0.05$). The Dj genetic differentiation showed a slightly higher value (Dj = 0.3303) than F_{ST} values and also with no significant difference ($P > 0.05$). Pairwise F_{ST} values were 0.084 between Osorno and Chiloé, 0.068 between Osorno and Llanquihue, and 0.052 between Chiloé and Lanquihue, and it did not significantly differ between populations ($P > 0.05$).

Results from the STRUCTURE assignment, based on ΔK Evanno's index, strongly supported K = 2 for the entire dataset. Q-values among populations were relatively symmetric (0.45–0.55) when assigned to two clusters (Fig. 3).

Table 3. Values of genetic fixation and differentiation calculated for pudu deer populations from southern Chile.

Loci	F_{ST}	P	Standardized F_{ST}	P	Dj	P
RT27	0.1626	0.066	0.2916	0.063	0.2778	0.077
N	0.0546	0.821	0.2562	0.830	0.3222	0.739
BM6506	0.0608	0.739	0.1622	0.746	0.2389	0.729
BM203	0.0756	0.462	0.3497	0.445	0.4097	0.456
BBJ2	0.1174	0.290	0.2788	0.327	0.4028	0.189
Mean	0.0942	0.472	0.2677	0.585	0.3303	0.417

F_{ST} (Wright 1965), the standardised F_{ST} (Hedrick 2005), population differentiation Dj (Gregorius and Roberds 1986).

Figure 3. Results of STRUCTURE analysis for K = 2 for the clustering model with population information included in the prior. The color indicates the relative membership proportion to each inferred genetic cluster based on Q-values. Sampled populations are separated by a white line.

Thus, the symmetric proportion of the sample assigned to each cluster reveals no population structure. In addition, the value of α was above 1 (α = 2.39–4.01), indicating that most individuals are admixed. Ne values as calculated with the LD method indicated the lowest Ne value for the Osorno population (Ne = 3) (Table 4). In Chiloé and Lanquihue populations, there was no evidence of variation of the genetic characteristic caused by genetic drift due to a finite number of parents. The comparison of different threshold values also shows that rare alleles do not have a great effect on the result for a given population.

Native forest fragmentation metrics

Results of five class metrics for forest cover revealed an important level of forest fragmentation in all analyzed provinces. However, the fragmentation level was higher in Chiloé Province than in Osorno and Llanquihue Provinces (Table 5). In fact, the PLAND was lower in Chiloé Province than in Osorno Province (22.3% and 37.1%, respectively), and concordantly, NP, PD, and SPLIT were all higher in this province. In addition, Chiloé Province showed lower patch area than Osorno and Llanquihue Provinces (SIZE = 61.20 vs. 98.59–245.78 ha), and approximately the half of size variation than those recorded in Osorno province (SIZE_CV = 384.35

Table 4. Effective population sizes (Ne) as calculated with NeEstimator based on the linkage disequilibrium model. For each population, Ne values are given for three different thresholds for the lowest allele frequency used. The values in parentheses are the 95% confidence intervals based on jack-knifing on loci.

		Effective population size (Ne)		
Population	**n**	**Frequency threshold 0.05**	**Frequency threshold 0.02**	**Frequency threshold 0.01**
Osorno	5	3 (0.5-infinite)	3 (0.5-infinite)	3 (0.5-infinite)
Chiloé	6	infinite (3.0-infinite)	infinite (3.0-infinite)	infinite (3.0-infinite)
Llanquihue	9	infinite (0.6-infinite)	infinite (0.6-infinite)	infinite (0.6-infinite)

n: sample size. "infinite" values of Ne refer to cases where there is no evidence of variation of the genetic characteristic due to finite numbers of parental individuals, i. e., all can be explained by sampling error (Do et al. 2014).

Table 5. Fragmentation metrics of native forest recorded in three localities from southern Chile. The metrics were calculated based on bounding rectangles of 12.2 × 12.2 km representing of pudu deer home range defined at each sampling site.

	Province		
Fragmentation metrics	**Osorno (n = 5)**	**Chiloé (n = 6)**	**Llanquihue (n = 9)**
Percentage of landscape (%)	37.13	22.36	18.64
Number of patches	86.60	107.16	66.33
Patch density (number of patches/100 ha)	0.58	0.71	0.44
Division	0.84	0.89	0.98
Split	289.3	966,270.8	442,787.7
Patch area (ha)	246.78	61.20	98.59
Patch area_CV (%)	537.61	384.35	322.54
Patch proximity	1,892.22	1,083.87	96.43
Patch proximity_CV (%)	121.62	108.09	140.44

n = number of bounding rectangles.

vs. 537.61%) (Table 5). The larger proximity index recorded for Osorno compared with Chiloé and Llanquihue Provinces (PROX = 1,892.22 vs. 96.43–1,083.87) also revealed that the native forest is less fragmented in this province.

Landscape genetic analysis

Sampling sites showed no significant spatial autocorrelation ($P > 0.05$) according to Moran's I index, both longitudinally (I = 1.4460, $P = 0.1215$) and latitudinally (I = 0 .3571, $P = 0.6597$). Therefore, these results indicate that the sampling sites were randomly distributed in the geographical area analysed. The model summary of the stepwise multiple regression revealed a significant and positive correlation with medium-high values between the response variables, represented by A and H_o, and the independent variables PD, PLAND and PROX (R = 0.6551, $P < 0.05$). In both cases, adjusted R^2 indicated that 32.22% of the variations in the response variable occurred because of changes in the independent variables. Furthermore, the results indicated significantly negative correlations between the number of alleles per locus and two independent variables represented by PD (β = -0.6594, $P < 0.05$) and PLAND (β = -0.8740, $P < 0.05$) (Table 6). Bootstrap correction of P-values showed that the correlations between A and PD ($P = 0.0123$, $P_{BOOTS} = 0.028$), and between A and PLAND ($P = 0.0151$,

Table 6. Final stepwise multiple regression models. Models show the effect of fragmentation metrics on the number of alleles per locus and the observed heterozygosity in pudu deer from southern Chile.

Parameter	Model	Coefficients ± SE	β ± SE	t-value	P
Number of alleles per locus	Intercept	1.7128 ± 0.0994			**0.0000**
	PD	-0.3889 ± 0.1379	-0.6594 ± 0.2338	-2.8206	**0.0123**
	PLAND	-0.0103 ± 0.0038	-0.8740 ± 0.3212	-2.7214	**0.0151**
	PROX	0.1244 ± 0.0710	0.5940 ± 0.3390	1.7525	0.0988
Observed heterozygosity	Intercept	0.7128 ± 0.0994			**0.0000**
	PD	-0.3889 ± 0.1379	-0.6594 ± 0.2338	-2.8206	**0.0123**
	PLAND	-0.0103 ± 0.0038	-0.8740 ± 0.3212	-2.7214	**0.0151**
	PROX	0.1244 ± 0.0710	0.5941 ± 0.3390	1.7526	0.0988

PD = patch density, PLAND = proportion of the landscape covered by forest, PROX = proximity index, SE = standard error of the estimate, β = regression coefficient. Bold indicate the significant values (P < 0.05).

P_{BOOTS} = 0.034) were statistically significant at $P < 0.05$. Similar results for *Ho* were found (Table 6). Therefore, this result suggests that PD and PLAND are variables associated with the reduction in genetic variation in pudu deer.

Discussion

Pudu deer populations in southern Chile are severely threatened by forest fragmentation (Echeverría et al. 2006, 2007; Lara et al. 2012; Miranda et al. 2017), a process that could shape its genetic structure. Nevertheless, the effects of fragmentation on the genetic features of pudu deer in populations distributed across its distribution range have never been investigated. In this study, we determined for the first time the genetic differentiation of three pudu deer populations living in a fragmented landscape by using microsatellite loci. Particularly, we focused our examination on the description of the genetic variability, genetic structure, and its association with the level of landscape fragmentation found at the analyzed sites.

The diversity genetic parameters displayed by the studied populations of pudu deer are comparable to those found in populations of this species distributed in other sites in southern Chile. Indeed, Cabello (2019), by using a different set of microsatellite markers to examine pudu deer from Concepción, Valdivia, Llanquihue, and Chiloé Provinces, found a mean observed heterozygosity of 0.470, a value that is within the range observed in our study (Ho = 0.423–0.547). The obtained genetic diversity values are also in the middle range as those obtained for other neotropical cervids using microsatellite markers. For example, the mean observed heterozygosity recorded in huemul deer (*Hippocamelus bisulcus*) (Corti et al. 2011) was 0.34, and in pampas deer (*Ozotoceros bezoarticus*) (Mantellatto et al. 2017) and in five subspecies of white-tailed deer (*Odocoileus virginianus*) (De La Rosa-Reyna et al. 2012), this parameter showed a value of 0.72 and from 0.53 to 0.64, respectively. Our analyses also showed higher values of homozygosity, indicating a higher degree of inbreeding within each population that is suggested by high F_{IS} values and the departure from the HWE of some loci. Although these results could be determined by intrinsic characteristics of the species rather than habitat fragmentation, our landscape genetic analysis provides evidence in favor of the habitat fragmentation because two fragmentation metrics were significantly associated with the genetic variation. In particular, the metrics of patch density and the proportion of the landscape covered by native forest had a negative relationship with the number of alleles per locus and the observed heterozygosity, suggesting that the habitat fragmentation may contribute to decreasing the genetic variation.

No significant difference in the F_{ST} values between populations observed in this study indicates no population genetic structuring in the dataset. This result is consistent with STRUCTURE analysis because the model did not detect population genetic structuring because no genetic clusters were inferred from the data set. In fact, the proportion of the sample assigned to each population represented by Q-values was roughly symmetric, revealing no population structure and with most individuals greatly admixed. Thus, this result suggests that there is gene flow between the study populations. It is known that STRUCTURE analysis may be affected by a smaller number of individuals or loci (Evanno et al. 2005, Kalinowski 2011); therefore, results of this study should be treated with caution. However, this result concurs with previous reports that show a low level of divergence of continental populations of pudu deer from Chile that in this study

correspond to Osorno and Llanquihue, but not with the evidence of the clear separation of Chiloé island's population from continental populations (Fuentes-Hurtado et al. 2011; Colihueque et al. 2022). For example, by using mtDNA control region and cytochrome b markers, Fuentes-Hurtado et al. (2011) found significant genetic structure in pudu deer from Chile, with two main divergent clades corresponding to continental and Chiloé island groups. Colihueque et al. (2022), using the same markers that considered the analysis of five populations located at different latitudinal coordinates and applying a cluster analysis, also found a distinctive cluster for Chiloé island individuals. Both studies, however, showed a lower level of genetic differentiation among continental populations than among continental and Chiloé island populations (F_{ST} = 0.153–0.168 vs. 0.590, and sequence divergence = 0.9% vs. 2.3%, respectively). These studies also indicate that the Chiloé island population shares some mtDNA haplotypes with continental populations, for example, the haplotype A of the cytochrome b gene (Colihueque et al. 2022), which suggests the occurrence of gene flow between the two geographical areas, a finding that concurs with the results of this study.

The results of this study are in accordance with research performed in deer species from other countries that indicates a significant influence of landscape features on the genetic structure of these mammals. For example, in roe deer (*Capreolus capreolus*) from France, fragmented woodland areas and landscape connectivity influence the gene flow of this species (Coulon et al. 2004). Breyne et al. (2014) found further evidence in roe deer from Flanders, as habitat fragmentation and anthropogenic barriers had a strong explanatory power for the observed genetic variation. In fact, physically proximate populations separated by anthropogenic barriers were more strongly differentiated from each other than those without such barriers in the landscape matrix. In the red deer (*Cervus elaphus* L.) from France and Croatia, the effect of habitat fragmentation on population genetic differentiation has also been supported (Dellicour et al. 2011; Sprem et al. 2013). Thus, results of this study are consistent with the general pattern of reduction in genetic variation of deer species living under conditions of high habitat fragmentation, a genetic effect that has also been documented in many mammals (Lino et al. 2019).

The heterozigosity deficit and the high number of private alleles observed in the studied populations may have different explanations, which include the presence of null alleles and the Wahlund effect. We discard the presence of null alleles in our dataset that could explain this deficit because the MICROCHECKER analysis did not provide support of their presence. However, we cannot exclude the existence of an underlying genetic structure in the study populations as a result of the Wahlund effect, which increases the probability of producing loci in a homocygous condition in small panmictic and isolated populations, due to the existence of population subdivision. However, the low level of genetic differentiation observed among the analyzed populations provides low support for this explanation because positive correlation between heterozigosity deficit and F_{ST} in strongly subdivided populations it is usually expected (De Meeûs 2018). Therefore, a more likely explanation could be related to the existence of relict populations of pudu deer living in fragmented habitats because this landscape modification gives rise to important allelic reductions, as it has been observed in other deer species (Zachos et al. 2007; Fernández-de-Mera et al. 2009; Dellicour et al. 2011, Sprem et al 2013). In addition, in species that exhibit high forest

dependency the fragmentation will have a more severe effect on the genetic variation as indicated by the ecological evidence gathered in numerous vertebrates (Vetter et al. 2011). In southern Chile, the forest fragmentation began several years ago, a process that was associated with the conquest and the colonial period starting in 1550 (Lara et al. 2012), which gave rise to many forest patches of variable size, especially in the central valley (Echeverría et al. 2008; Miranda et al. 2017). In this study, we corroborated that at the study site, the forest fragmentation was relatively intense, as was revealed by the high number of patches and patch density, especially in Chiloé province. In this context, and taking into account that the pudu deer can be considered a mammal with high forest dependency (Jiménez 2010), the heterozigosity deficit observed in the populations under analysis is consistent with the potential presence of relict populations at the study sites. These populations may have lost genetic variability over generations as a result of random drift and inbreeding, given their small population effective size. Our results suggest that at least one studied population may have experience further genetic variability reduction as a consequence of their low Ne because it was considerably smaller than the theoretically tolerable threshold value (Ne = 50, representing heterozygosity reduction of 1% each generation, Frankham et al. 2002). This result is in line with studies of other deer species influenced by anthropogenic factors, such as the European red deer, where some populations with reduced Ne have been reported (Zachos et al. 2016). However, since in our case the calculated Ne values could be biased due to the small sample size, this conclusion should be taken with caution. Considering this limitation, the possible presence of pudu deer populations with reduced Ne can have conservation implications because the increase in homozygosity and the concomitant effects of recessive deleterious alleles may eventually result in a reduction of reproductive fitness of individuals, giving rise to an inbreeding depression (Frankham et al. 2002). This phenomenon may reduce the long-term survival of the pudu deer population. In fact, there are studies that provided evidence on the occurrence of this phenomenon in wild populations of red deer because the presence of individuals with morphological malformations, which are sign of inbreeding depression, have been related to low levels of genetic variation (Zachos et al. 2007). Further analysis considering a large number of populations of pudu deer inhabiting areas with variable levels of fragmentation, including a large sample size, as the number of individuals sampled per site is an important consideration (Wang 2011; Hale et al. 2012; Landguth et al. 2012), will be required to add more support about the possible existence of heterozygosity reduction in pudu deer populations distributed in southern Chile. In other deer species, the analysis of populations living in a variable level of landscape fragmentation has been a useful method to clarify the effect of fragmentation on the genetic structuring of populations (Dellicour et al. 2011; Breyne et al. 2014). Another approach, for example, by comparing the genetic structure of populations living in continuous and fragmented landscapes, could also be considered because this design has been shown to be effective in addressing this problem in mammals (Bani et al. 2017).

The findings of this study have conservation implications for pudu deer because habitat fragmentation is one of the major concerns for the long-term conservation of this native mammal in our country (Jiménez 2010). In our case, it was found that such habitat modification affects the genetic variation and the structure of the species, which may reflect a reduction or interruption of

gene flow among populations, possibly turning populations genetically more structured and less diverse, as has been observed in small mammals living in strongly fragmented landscapes (Brunke et al. 2020). Therefore, it is of great importance to maintain the forest continuity across the distribution range in southern Chile for the preservation of the genetic diversity of pudu deer. Currently, this objective is relevant because relatively well-preserved native forest that provides suitable habitat for pudu deer is largely under-represented in national protected areas (Pavez-Fox and Estay 2016). In this context, we suggest making special efforts targeting native forest maintenance and reforestation as a strategy to reduce the effect of fragmentation on the genetic diversity of extant populations of pudu deer. This strategy could reduce the existence of a hostile habitat characterized by a mixture of small patches of different sizes and isolation surrounded by a matrix of unsuitable habitat that limits the movement of individuals, as it represents barriers that constrain the dispersal of individuals and, as a consequence, affect their spatial distribution in the landscape (Manel et al. 2003; Storfer et al. 2007). Other strategies, such as those that consider the presence of corridors, could also be appropriate for increasing gene flow among populations because they can reduce isolation by improving population connection and therefore, increase the likelihood of long-term survival of populations (Burkart et al. 2016). These strategies likely will contribute to improving the conservation status of pudu deer, which is currently classified as nearly threatened with a decreasing population trend according to the IUCN.

Conclusions

The genotyping with microsatellite loci of georeferenced individuals of pudu deer from three populations of southern Chile revealed genetic diversity values comparable to those found in populations of this species distributed in other sites of this geographical area. There was a low genetic differentiation among populations and significant deficits of heterozygotes, revealing a genetic variation loss that may have occurred as a consequence of a decrease in population effective size, genetic drift, or the impairment of gene flow among populations. Correlation analyses between native forest fragmentation and genetic variation parameters identified two fragmentation metrics, particularly the proportion of the landscape covered by forest and the patch density, with a negative and significant correlation with the number of alleles per locus as well as with the observed heterozygosity. This result suggests that the degree of habitat fragmentation may contribute to decreasing the genetic variation of pudu deer. Maintaining the native forest continuity across the distribution range of pudu deer could contribute to preserving the genetic diversity of this mammal, enhancing the long-term survival of this mammal.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: NC. Formal analysis: NC. Funding acquisition: NC. Methodology: NC. Writing - original draft: NC. Writing - review and editing: AG.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Microsatellite loci

Authors: Nelson Colihueque, Alberto Gantz

Data type: pdf

Explanation note: Polymorphic microsatellite loci used in this study. The level of polymorphism of each locus, measured as number of alleles, allele size range and observed heterocigosity, were obtained from Marín et al. (2014) based on genotyping data of pudu deer samples. The original species in which microsatellite loci were isolated are also shown, with the accession number and chromosome location.

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Link: https://doi.org/10.3897/neotropical.20.e151162.suppl1

Supplementary material 2

Primers sequences

Authors: Nelson Colihueque, Alberto Gantz

Data type: pdf

Explanation note: Primers sequences and PCR conditions used for amplification of polymorphic microsatellite loci in pudu deer.

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Link: https://doi.org/10.3897/neotropical.20.e151162.suppl2

Supplementary material 3

Allele frequencies

Authors: Nelson Colihueque, Alberto Gantz

Data type: pdf

Explanation note: Allele frequencies of five microsatellite loci for pudu deer of three populations from southern Chile.

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